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CYTOLOGIC EFFECTS OF AIR FORCE CHEMICALS (Third of a Series)

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

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Studies of DNA replication and repair in cell cultures have shown that hydrazine, although highly toxic to cells, does not damage DNA and thus interfere directly with DNA replication in Chinese hamster ovary cells grown in vitro, nor does it affect DNA repair synthesis in CCL-185 human lung cells grown in vitro. Hydrazine does not inhibit the methylation of DNA by the direct acting alkylating agent methylmethanesulfonate (MMS) in lungs

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Mice were injected with naphthalene and bromobenzene and elevated levels of DNA replication and repair were observed in lung slices tested in vitro. This may be the result of sensitive cells in the lung being killed by the chemicals and the survivors replicating with an increased capacity for repair while they were proliferating. In a comparable experiment, treatment of mice with hydrazine was found to only marginally increase lung cell replication.

Peripheral lymphocytes from human blood were exposed to carcinogens in vitro and DNA repair synthesis was measured. Exposure to 4NQO, MMS, nitrogen mustard or MMC, all known DNA damaging agents (although each with a different mode of action), induced DNA repair activity. This repair activity was greatly amplified when lymphocytes were stimulated to divide with phytohemagglutinin.

SUMMARY

Studies of DNA replication and repair in cell cultures have shown that hydrazine, although highly toxic to cells, does not damage DNA and thus interfere directly with DNA replication in Chinese hamster ovary cells grown in vitro, nor does it affect DNA repair synthesis in CCL-185 human lung cells grown in vitro. Hydrazine does not inhibit the methylation of DNA by the direct acting alkylating agent methylmethanesulfonate (MMS) in lungs of mice exposed in vivo, nor does it inhibit the removal of methylated DNA bases.

A method has been developed for treating lung slices of rats and mice in vitro with chemicals and monitoring their effect on DNA replication and repair. This method has been used to show that the alkylating agents MMS and 4-nitroquinoline-1-oxide (4NQO), both mutagens and carcinogens, induce DNA repair in both rat and mouse lung tissue after in vitro exposure.

Mice were injected with naphthalene and bromobenzene and elevated levels of DNA replication and repair were observed in lung slices tested in vitro. This may be the result of sensitive cells in the lung being killed by the chemicals and the survivors replicating with an increased capacity for repair while they were proliferating. In a comparable experiment, treatment of mice with hydrazine was found to only marginally increase lung cell replication.

Peripheral lymphocytes from human blood were exposed to carcinogens in vitro and DNA repair synthesis was measured. Exposure to 4NQO, MMS, nitrogen mustard or MMC, all known DNA damaging agents (although each with a different mode of action), induced DNA repair activity. This repair activity was greatly amplified when lymphocytes were stimulated to divide with phytohemagglutinin.

PREFACE

This is the fourth annual report of the Cytology, Cell Biology and Cytogenetics Section of the Toxic Hazards Research Program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract F33615-76-C-5005. This report describes the research activities at UCI from July, 1979 through June, 1980. During this period, T.T. Crocker, M.D. was Principal Investigator for the contract. R.E. Rasmussen, Ph.D. conducted the studies at UCI. Jean Anderson served as Staff Research Associate and Arthur T. Fong as Research Assistant at UCI.

This subprogram is concerned with the relationship of cytogenetic effects of environmental chemicals to their toxic or carcinogenic effects in experimental animals. To this end studies include chromosome damage, mutagenesis, DNA synthesis and repair, and cellular proliferation in vivo and in vitro.

The goal of the subprogram is to better understand the mechanisms by which carcinogenic or mutagenic chemicals cause genetic damage, and to thereby provide means of evaluating possible hazards of exposure to new materials entering the environment.

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CELL CULTURE STUDIES WITH HYDRAZINE

INTRODUCTION

Hydrazine is toxic to mammalian cells in culture and this toxicity can be measured in a number of ways. In a previous report (Benz et al., 1979), we have shown effects on DNA replication in several cell lines. The effect on DNA replication of a 1 hr exposure to hydrazine was inhibition followed by recovery of the normal rate of replication. The additional studies reported here are concerned with two possible effects of hydrazine: 1) the mechanism of DNA replication inhibition, and 2) the possible effect of hydrazine on DNA repair processes.

METHODS AND MATERIALS

Cell cultures were grown in commercially prepared cell medium (Eagle's Minimal Essential Medium with non-essential amino acids and fetal bovine serum, GIBCO). ^{3}H-thymidine (^{3}H-TdR) was from Schwarz/Mann.

To determine the thymidine labeling index after treatment with hydrazine, Chinese hamster ovary (CHO) cells were seeded into 60 mm plastic dishes (3×10^{5} cells per dish) that contained a glass coverslip. Twenty-four hours later, the growth medium was removed from all the dishes and to one-half, phosphate buffered saline (PBS) at pH 4.5 containing 15 mM hydrazine was added. The remaining dishes received PBS with no additions. We have found, using chemical assay methods, that hydrazine is stable in PBS at pH 4.5 for several hours. At the end of 1 hr, all dishes were returned to normal growth medium. At various times after treatment, cultures were labeled with ^{3}H-TdR (5 μCi/ml) for 10 min, fixed with acetic acid:ethanol (1:3) and prepared for autoradiography.

For autoradiography, coverslips bearing cells were attached cell-side-up to microscope slides, dipped in Eastman NTB-2 emulsion (diluted 1:1 with water) and allowed to expose for several days at -20°C before development with Kodak D-19. The preparations were stained with Giemsa and the fraction of labeled cells was determined by counting all cells in randomly selected microscopic fields and recording the number with silver grains over nuclei. At least five hundred cells were counted on each coverslip.

To test for the effect of hydrazine on ultraviolet (UV) light induced DNA repair, a human cell line (CCL-185, American Type Culture Collection) derived from a human lung carcinoma, was used. This cell line was used in preference to the CHO cell line because it shows a much greater DNA repair response to UV-induced DNA damage, and the repair is thus more readily measured. For these experiments, the cells were seeded into 60 mm diameter plastic dishes which contained coverslips. At 48 hr after seeding, the medium was removed and replaced with 2.0 ml of PBS. One-half of the dishes were exposed to 20 J/m^{2} of UV light from a 4-watt low pressure mercury arc (Mineralite, Ultraviolet Products Inc., San Gabriel, California). All dishes were returned to normal growth medium containing ^{3}H-TdR (5 μCi/ml). One-half of the control and one-half of the UV exposed dishes also contained 15 mM hydrazine. At intervals after UV exposure,

coverslips were removed from three dishes of each group, fixed, and mounted cell-side-up on microscope slides. Autoradiographs were prepared as described above.

RESULTS AND DISCUSSION

Treatment of CHO cells with 15 mM hydrazine for 1 hr reduced the fraction of cells incorporating ^{3}H-TdR into nuclear DNA from about 50% to 18% (Table 1). By 4 hr, the fraction of cells labeled had increased substantially and by 8 hr was not different from the control level. This result suggests that, although hydrazine is clearly toxic and can interfere with cellular metabolism, it does not cause damage to nuclear DNA which can interfere with subsequent replication of the DNA as judged by ^{3}H-TdR incorporation. These results provide little information on the possible mutagenic effects of hydrazine, but they do show that the toxic effect of a short exposure to hydrazine briefly stops DNA replication after which the normal replication level is resumed. The results presented are also in general agreement with those reported previously (Benz et al., 1979) in which the rate of ^{3}H-TdR incorporation was depressed only as long as hydrazine was present and then returned to normal levels when hydrazine was removed.

These studies should not be considered as predictive of effects with the methylated hydrazines; the latter have been shown to be mutagenic and carcinogenic in vitro and in vivo. Hydrazine itself may also have a role in carcinogenesis; however, the mechanisms are obscure. The above studies show only that hydrazine at relatively high concentrations can inhibit DNA replication, but not whether this may contribute to mutagenesis or carcinogenesis.

Table 1. Thymidine labeling index in CHO cells after a 1 hr treatment with 15 mM hydrazine *

Time After Hydrazine Exposure	Control	Hydrazine Treated
1 hr	0.50	0.18
4 hr	0.51	0.44
8 hr	0.41	0.60
12 hr	0.64	0.67
24 hr	0.51	0.63
28 hr	0.49	0.61
32 hr	0.56	0.64

* Values are the fraction of cell nuclei that are labeled, and are the average of counts of 2 coverslips of at least 500 cells per coverslip.

The possibility that hydrazine might inhibit the repair of DNA damage was studied in a human cell line derived originally from a lung carcinoma and presumed to have been the result of carcinogenic transformation of a type II alveolar cell. Cultures of these cells (designated type CCL-185) were exposed to UV light (20 J/m^{2}) and then incubated with or without 1 mM hydrazine and ^{3}H-TdR. During DNA repair, ^{3}H-TdR is incorporated into nuclear DNA and can provide a quantitative measure of the amount of

repair. At intervals following the UV light exposure, cell cultures were fixed and prepared for autoradiography as described in the Methods and Materials. DNA repair activity was quantified by counting the silver grains in the photoemulsion over cell nuclei. The results presented in Table 2 clearly show that hydrazine had no effect on DNA repair synthesis, and further that hydrazine did not induce DNA damage, the repair of which involved incorporation of ^{3}H-TdR.

Table 2. A test for the effect of hydrazine on UV light induced DNA repair in cell line CCl-185*

	Control		UV Exposed	
Time After UV Exposure	Without Hydrazine	With Hydrazine	Without Hydrazine	With Hydrazine
1 hr	1.23	1.39	3.89	4.53
2 hr	1.51	2.16	8.66	9.17
4 hr	4.06	5.13	14.25	14.77
6 hr	5.88	4.71	18.64	18.16

* Values are the average number of grains per cell nucleus based on counts over at least 150 cell nuclei per coverslip.

These studies provide only a limited amount of information on the possible effects of hydrazine on DNA repair. A more direct study is presented below in which in vivo effects of hydrazine on DNA damage produced by an alkylating agent are measured.

IN VIVO EFFECTS OF HYDRAZINE ON DNA METHYLATION IN THE MOUSE BY METHYLMETHANESULFONATE

INTRODUCTION

Methylhydrazines can serve as methyl donors for the alkylation of DNA in vivo. This is evidence that hydrazines can, at some point, influence or enter the metabolic pathways which regulate DNA methylation. The precise role of DNA methylation is unknown but may be related to regulation of transcription. Also of great interest is the observation that treatment of rats or mice with hydrazine or certain other toxins results in an increased methylation of DNA bases in the liver. Since hydrazine has no methyl groups, it must be affecting (or effecting) methylation of the DNA bases in an indirect manner. Two major hypotheses are that 1) hydrazine is methylated in vivo and then can act as a methyl donor, and 2) hydrazine stimulates or induces an endogenous DNA methylase which leads to an increase in the average level of DNA methylation (Shank, 1979). A third speculation is that methylation and demethylation (or repair) are normal cellular activities and that hydrazine (or other stressors) may inhibit the demethylation/repair step to a somewhat greater extent than the methylation.

A test of this hypothesis could be to introduce a methylating agent which is independent of metabolic activation, and then to monitor the removal of methylated bases from DNA with time after treatment in animals exposed (or not) to hydrazine. If hydrazine inhibits demethylation/repair, then treated animals should show a slower removal with time of methylated bases in DNA.

These studies were conducted with the collaboration of Dr. R.C. Shank and his associates Ms. Deborah Herron and Mr. Louis R. Barrows.

METHODS AND MATERIALS

To determine the binding of ^{14}C-methylmethanesulfonate (^{14}C-MMS) to liver DNA of hydrazine treated mice, male C57B1/6J mice, approximately 2 mo old, were injected intraperitoneally (ip) with hydrazine dissolved immediately before use in 0.9% NaCl to give a dose of 10 mg/kg body weight. Control animals received 0.9% NaCl only. Immediately after the hydrazine, all animals were injected with ^{14}C-MMS (10 μCi/mouse, 10 mCi/mmole, in 0.9% NaCl). Triplicate animals from hydrazine and control groups were killed at 1, 4, 24 and 48 hr. DNA was extracted from lung and liver using a modified Marmur procedure involving homogenization and lysis of the tissue with detergent, precipitation of the protein with $CHCl_3$ and digestion with RNase (Marmur, 1961). The yields of DNA were determined by the diphenylamine procedure (Schneider, 1957) and absorbance measurements. Specific radioactivity was determined by scintillation counting.

The DNA samples were further analyzed to obtain information on the amounts of methylated guanine residues in the DNA. The DNA samples were hydrolyzed and chromatographed using high pressure liquid chromatography (HPLC). The proportions of the methylated guanines were calculated on the basis of the weight of DNA applied to the column and the amount of radioactivity recovered in the fractions eluting from the column that contained authentic 7-methylguanine and O^6-methylguanine (Herron and Shank, 1979).

RESULTS AND DISCUSSION

The specific radioactivity of the liver DNA samples differed little between the hydrazine treated and control mice except for the value obtained for the 48 hr sample from the hydrazine treated group. This high value may not be due to binding of MMS to DNA but rather to some unrecognized contaminant. The further analysis of the binding of ^{14}C-MMS to DNA guanine presented below tends to support this idea, since the binding of ^{14}C-MMS to DNA guanine from the hydrazine treated mice was not higher than the controls at 48 hr posttreatment. The yields of DNA from the mouse lungs were too small to provide reliable measurements of the specific radioactivity.

Table 3. Binding of ^{14}C-MMS to liver DNA of hydrazine treated mice

Sample Time	Treatment	^{14}C-DPM/mg DNA	µmoles/mole G*
1 hr	Control	285	20.8
1 hr	Hydrazine	241	17.6
4 hr	Control	269	19.6
4 hr	Hydrazine	280	20.4
24 hr	Control	467	34.1
24 hr	Hydrazine	319	23.3
48 hr	Control	430	31.4
48 hr	Hydrazine	934	68.2

* Calculated from the specific radioactivity of the ^{14}C-MMS.

Alkylation of DNA by agents such as MMS seems to occur predominantly at the 7 position of guanine, and much less frequently at other sites. The alkylation of guanine in the mouse liver DNA was examined as described in the Methods and Materials and the results are given in Table 4. This analysis was done on the same DNA samples for which the specific radioactivity was determined in Table 3. The results indicate that hydrazine treatment produced no significant difference in DNA methylation compared to controls.

Table 4. Quantitation of methylated guanines in liver DNA of mice treated simultaneously with hydrazine and ^{14}C-MMS*

Time After Treatment	Control		Hydrazine	
	7-MeG	O^6MeG	7-MeG	O^6MeG
1 hr	13.29	0.55	9.85	0.15
4 hr	9.00	0.00**	11.9	0.92
24 hr	3.12	0.31	4.45	0.27
48 hr	5.16	0.12	4.26	0.19

* The values are micromoles of methylated base per mole of guanine.
** No radioactivity detected.

The specific radioactivity measured (Table 3) and direct determination of methylated guanines (Table 4) indicate that about one-half of the total alkylation is on guanine for the 1 hr and 4 hr samples. At 24 hr and 48 hr, the overall specific radioactivity measurement remained high but the alkyl guanines are reduced by half. DNA repair may account in part for the loss of alkyl guanines, but the sites of the remaining ^{14}C label are unknown at this time.

DNA REPLICATION AND DNA REPAIR SYNTHESIS IN RODENT LUNG AFTER TREATMENT WITH LUNG TOXINS

INTRODUCTION

Inhalation of various toxic materials is known to cause damage to cells of the respiratory tract and to thereby elicit cellular proliferation of some cell types and infiltration by macrophages into the alveolar regions (Matulionis, 1979). Cellular proliferation in vivo or in vitro can be followed and quantified by using radiotracers (e.g. ^{3}H-TdR) combined with autoradiography. A more indirect measurement of cell proliferation can be made using radiochemical determination of ^{3}H-TdR incorporation into lung slices in vitro.

Under separate support the above methods have been used to study DNA replication and DNA repair synthesis in lungs of mice chronically exposed to cigarette smoke. It was found that chronic smoke exposure caused an elevation in DNA replicative synthesis, and a reduction in the DNA repair (Rasmussen et al., 1979). Autoradiographic studies have suggested that an alteration in the DNA repair capacity of bronchiolar epithelial cells has occurred. The relationship of these findings to lung cancer is currently under investigation.

Of possible interest to the Air Force are the findings that certain common hydrocarbons such as naphthalene (Mahvi et al., 1977) and halogenated benzene (Reid et al., 1973) may selectively damage groups of lung cells. Present evidence suggests that these materials are metabolically converted to toxic intermediates by the nonciliated epithelial cells (Clara cells) of the terminal bronchioles of rodents. At relatively high doses, the Clara cells are selectively killed and slough into the lumina of the airways. If the animals survive, the Clara cell population is restored by proliferation of stem cells.

Because aircraft fuels and fuel additives may contain naphthalene or related hydrocarbons, something should be known of the possible damage to the lung and sequelae that may occur after an acute exposure. To this end, in vitro studies have been conducted of DNA replication in lungs of rats and mice, and studies of the DNA repair response after in vitro challenge of the tissue with DNA alkylating agents. These studies are providing information on DNA replication and repair which may be compared to data obtained in planned future studies of the effect of acute, high level hydrazine exposures in rats and hamsters.

METHODS AND MATERIALS

Young adult male Fischer 344 rats or C57Bl/6J mice, 2-3 mo old, were anesthetized with Nembutal and exsanguinated by cutting the femoral arteries. After removing the ventral skin, the thoracic cavity was laid open by carefully cutting through the rib cage on either side to expose the lungs and trachea. A short length of polyethylene tubing was inserted into the trachea through a small slit between the tracheal rings and tied in place. The lungs were then fully inflated through the tubing with warm (42°C) 2% agar in a balanced salt solution, and the tubing removed and the trachea ligated. The lungs, together with the trachea and heart, were then dissected free and placed in ice cold salt

solution. After thorough chilling, the lobes of the lung were cut free from the heart and trachea and placed in the barrel of a 5 ml plastic syringe, the needle end of which had been cut off and replaced with the plunger. The syringe was then completely filled with warm 2% agar and allowed to solidify around the lung lobes. After chilling, the cylinder of agar containing the lung tissue was gradually extruded from the syringe using the plunger, and slices of approximately 1 mm thickness were cut freehand and accumulated in ice cold buffered salt solution.

The lung slices were exposed in vitro to either MMS or 4-nitroquinoline-1-oxide (4NQO) at the concentrations indicated in the Table legends in order to produce DNA damage in the lung cells and to stimulate DNA repair synthesis. The latter was quantified by simultaneously incubating the slices with ^{3}H-TdR (5 µCi/ml, 60 Ci/mmole) in a nutrient medium consisting of Dulbecco's phosphate buffered saline supplemented with essential amino acids and 1% fetal bovine serum. In order to suppress replicative DNA synthesis but not repair synthesis, hydroxyurea (HU) was also added at a concentration of 10 mM. After incubation at 37°C the specific radioactivity of the DNA in the slices was determined using a chemical extraction procedure (Scott et al., 1956). DNA repair synthesis was calculated by subtracting the specific radioactivity of DNA found in lung slices incubated with HU and ^{3}H-TdR, but without MMS or 4NQO, from that found when one of the alkylating agents was present. For measurement of normal replicative DNA synthesis in the slices, both HU and the alkylating agents were omitted during the incubation with ^{3}H-TdR.

RESULTS AND DISCUSSION

DNA Replication and Repair Synthesis in Rat Lung

Time dependence of DNA replicative synthesis and DNA repair synthesis resulting from treatment of rat lung slices with 1 mM MMS are shown in Table 5. The results indicate that DNA synthesis and repair are very active in the lung slices and that this method is applicable to the study of these activities in rats as well as in mice as demonstrated previously.

Table 5. DNA replication and repair synthesis in rat lung tissue*

Time of Incubation	DNA Replication	DNA Repair Synthesis
1 hr	5048 ± 1791	69 ± 36
2 hr	6877 ± 1495	864 ± 318
4 hr	20308 ± 1878	1111 ± 330

* Slices of agar inflated rat lung were incubated in vitro with 1 mM MMS, 5 µCi/ml ^{3}H-TdR and with or without 10 mM HU. The values are ^{3}H-DPM/µg of DNA and are the average of triplicate samples ± S.D.

DNA Replication and Repair Synthesis in Mouse Lung

Using the methods described above, DNA replication and repair synthesis have been measured in mouse lung slices after treatment in vitro with MMS and 4NQO. Table 6 shows the results of a dose response study with MMS. At 1 mM, DNA replication was depressed by about 50% and DNA repair synthesis was quite active. Higher concentrations of MMS were not tested. A time study of MMS induced DNA repair is shown in Table 7. In agreement with previous studies, the rate of DNA repair synthesis was greatest during the first 2 hr after treatment.

Table 6. DNA repair synthesis induced in mouse lung in vitro by MMS*

Concentration of MMS	Control	10 mM HU	Net DNA Repair
0	2878 ± 205	224 ± 16	-
10 μM	2868 ± 630	254 ± 5	30 ± 8
100 μM	3135 ± 535	427 ± 61	203 ± 32
1 mM	1313 ± 88	467 ± 66	243 ± 34

* Lung slices were incubated in ^{3}H-TdR (5 μCi/ml) ± 10 mM HU at the indicated concentrations of MMS. The values are as in Table 5.

Similar studies with 4NQO are shown in Tables 8 and 9. This compound is a lung carcinogen in mice. The results show both time and concentration dependence of DNA repair synthesis induced by 4NQO.

Table 7. DNA repair synthesis with time of incubation with 1 mM MMS*

Time of Incubation	HU + MMS	HU Only	MMS Only	Control	(HU+MMS)-(HU) = Net DNA Repair
1 hr	304 ± 123	182 ± 82	817 ± 121	971 ± 19	122 ± 74
2 hr	509 ± 39	199 ± 24	1403 ± 114	1785 ± 142	310 ± 23
4 hr	773 ± 53	365 ± 26	1408 ± 109	3284 ± 146	417 ± 30

* Lung slices were incubated in vitro with 1 mM MMS, 5 μCi/ml ^{3}H-TdR and ± 10 mM HU. The values are as in Table 5.

Table 8. DNA repair synthesis induced in mouse lung in vitro by 4NQO*

Concentration of 4NQO	Control	10 mM HU	Net DNA Repair
0	6359 ± 239	495 ± 70	-
1 μM	5543 ± 1628	377 ± 59	0
10 μM	8332 ± 4872	807 ± 99	312 ± 61
100 μM	2408 ± 1078	720 ± 164	225 ± 89

* Lung slices were incubated for 4 hr in vitro with 4NQO and 5 μCi/ml ^{3}H-TdR ± 10 mM HU. The values are as in Table 5.

Table 9. DNA repair synthesis in mouse lung with time of incubation with 100 μM 4NQO*

Time of Incubation	HU + 4NQO	HU Only	4NQO Only	Control	(HU+4NQO)-(HU) = Net DNA Repair
1 hr	524 ± 82	217 ± 10	1238 ± 134	2591 ± 267	307 ± 41
2 hr	917 ± 31	310 ± 43	1501 ± 105	3877 ± 405	607 ± 27
4 hr	929 ± 98	514 ± 65	1704 ± 380	6279 ± 1165	415 ± 59

* Lung slices were incubated in vitro with 100 μM 4NQO, 5 μCi/ml ^{3}H-TdR and ± 10 mM HU. The values are as in Table 5.

Effect of Naphthalene and Bromobenzene on Lung DNA Synthesis

C57B1/6J male mice were injected i.p. with naphthalene in corn oil (250 mg/kg body weight). At 24, 72 and 168 hr, DNA replication and repair synthesis in lung slices were measured as described above. Table 10 summarizes the results. At 24 hr, there was little difference between treated and controls, but at 72 hr, the replicative DNA synthesis in lung slices from the treated mice was clearly greater than the controls, and DNA repair capacity was also significantly increased. In studies conducted with another mouse strain (BC3F1/Cum) under separate support, similar results were obtained and even at 14 days posttreatment, DNA replication and repair response were still elevated.

Table 10. DNA replicative and repair synthesis in lung tissue from C57B1/6J mice treated with 250 mg/kg body weight naphthalene dissolved in corn oil*

Treatment	Time Post-treatment	Replicative DNA Synthesis	DNA Repair Synthesis
Corn Oil	24 hr	3759 ± 360	311 ± 48
Naphthalene	24 hr	3154 ± 607	270 ± 26
Corn Oil	72 hr	3120 ± 189	388 ± 86
Naphthalene	72 hr	8890 ± 1941	523 ± 81
Corn Oil	168 hr	4288 ± 520	442 ± 71
Naphthalene	168 hr	14791 ± 1526	833 ± 60

* Lung slices were incubated 4 hr with ^{3}H-TdR (5 μCi/ml), ± 10 mM HU and ± 1 mM MMS. Values are ^{3}H-DPM/μg of DNA based on triplicate samples from each of two mice in each treatment group, ± S.D.

Taking into account the results of others, these results suggest that naphthalene has caused the loss of a group of lung cells which are subsequently replaced by replication of a precursor population, and the proliferation of these latter cells is the cause of the increased DNA replication seen in the lung. A possible explanation for the modest increase in DNA repair capacity is more speculative, but may also be related to the increased population of replicating cells induced by the naphthalene treatment. DNA repair synthesis capability seems to be greatest in cells that are actively proliferating compared to nonproliferating cell types (e.g., peripheral lymphocytes). Therefore, an increase in the proportion of replicating cells in the lung would be expected to increase the overall DNA repair capability. In the present case, the replicating population is the epithelial cells of the bronchioles. This cell population appears to be especially active in DNA repair as judged by the results of quantitative autoradiographic studies of lung tissues treated in vitro with DNA damaging agents. The epithelial cells of the bronchioles show about five times more incorporation of ^{3}H-TdR than the alveolar cells, i.e., type I and II cells. Confirmation that naphthalene or other lung toxins may elicit cell populations which are more (or less) competent in DNA repair must await the results of future studies.

The results obtained with mice treated with bromobenzene (BrBz) resembled those found with naphthalene. At 24 hr posttreatment with BrBz (5 mmoles/kg body weight), both DNA replication and DNA repair response in the BrBz treated mice were significantly increased over controls (Table 11), suggesting a burst of cellular replication of cells which are also repair competent. At 7 and 10 days, both DNA replication and repair capacity remained elevated. Because of the small numbers of animals, these results must be considered only preliminary.

Future Plans

Studies with both naphthalene and BrBz will be designed to extend for longer periods. The continuing rationale for these studies is that they will provide data on the response of the lung to toxic materials, against which the effects of test materials may be compared. An example of such a comparative study is presented in the next section.

Table 11. Effect of bromobenzene on DNA replication and repair synthesis in mouse lung*

	Corn Oil		Bromobenzene	
Time After Treatment	DNA Replication	DNA Repair	DNA Replication	DNA Repair
24 hr	2659 ± 531	654 ± 97	1423 ± 178	298 ± 32
72 hr	2199 ± 135	414 ± 61	16770 ± 1675	1048 ± 83
168 hr	4009 ± 147	588 ± 122	7966 ± 1241	619 ± 61
240 hr	6195 ± 419	663 ± 76	14745 ± 1931**	1478 ± 220**

* Lung slices incubated and values given as in Table 10.
** Values from one mouse only.

Comparison of the Effects of Naphthalene and Hydrazine on DNA Synthesis in Mouse Lung

Exposure to hydrazine may occur as the result of accidental release of hydrazine under pressure and relatively large amounts may be inhaled, ingested or absorbed through the skin. Experimental studies with animals exposed in this manner are in progress, and such animals will be studied using the methods described in this report.

A preliminary study has been done to compare exposure to hydrazine with that of naphthalene. C57B1/6J mice were injected ip with naphthalene dissolved in corn oil (0.06 ml of a 100 mg/ml solution) or with corn oil only. Hydrazine treated mice were injected ip with 0.1 ml of a 2 mg/ml solution of hydrazine in pH 4.5 0.9% NaCl prepared immediately before use, or with saline only. At selected times posttreatment, two mice of each group were killed and DNA replication and repair synthesis in agar inflated lung slices was measured in vitro. DNA repair synthesis was stimulated with 1 mM MMS. DNA replication was measured as the incorporation of ^{3}H-TdR into cellular DNA in the absence of MMS or HU. DNA repair synthesis was measured as the difference between the incorporation of ^{3}H-TdR into cellular DNA in the presence of HU when MMS was present and when it was absent.

At 10 days posttreatment, when the naphthalene treated mice were expected to show increased cell proliferation (i.e., DNA replication) in the lung, the animals were killed and DNA replication and repair were analysed as described. The results (Table 12) show the expected effect of naphthalene, and are suggestive in the case of the hydrazine treated mice. Both DNA replication and repair capacity were increased in the lung from naphthalene treated mice and DNA replication is slightly increased in the lungs of the mice treated with hydrazine.

An experiment to test the immediate effects of hydrazine on DNA replication in mouse lung was also done. Male C57B1/6J mice were injected with a solution of hydrazine or saline as above. One hour later, the mice were killed with an overdose of

sodium pentobarbital and DNA replication and repair synthesis in the lungs was determined as was described previously. There was no significant effect on either replication or repair capacity as shown in Table 13.

These studies are continuing and will be extended to include rats and hamsters to be exposed to hydrazine at the THRU.

Table 12. Comparison of effects of naphthalene and hydrazine on DNA synthesis in mouse lung*

	24 hr		168 hr	
Treatment	DNA Replication	DNA Repair	DNA Replication	DNA Repair
0.9% NaCl	3305 ± 490	654 ± 88	8640 ± 900	802 ± 128
Hydrazine	3263 ± 297	516 ± 69	3952 ± 928	411 ± 95
Corn Oil	4003 ± 901	598 ± 140	4155 ± 326	627 ± 61
Naph.	2695 ± 268	667 ± 68	27990 ± 5034	1044 ± 337

	240 hr	
Treatment	DNA Replication	DNA Repair
0.9% NaCl	4588 ± 675	787 ± 70
Hydrazine	7151 ± 1014	643 ± 47
Corn Oil	5021 ± 275	800 ± 138
Naph.	11580 ± 176	1108 ± 257

* Values are ^{3}H-DPM/μg of DNA and are based on triplicate determinations on each mouse. Error is ± S.D.

Table 13. A test for an immediate effect of hydrazine on DNA replication and repair synthesis in mouse lung*

Treatment	DNA Replication	DNA Repair
Saline	4953 ± 619	957 ± 234
Hydrazine	4795 ± 344	868 ± 145

* Values are based on triplicate determinations on two mice of each group and are ^{3}H-DPM/μg of DNA ± S.D.

DNA REPAIR IN PERIPHERAL LYMPHOCYTES

INTRODUCTION

The use of peripheral lymphocytes from humans and experimental animals as an indicator cell population for monitoring potentially harmful effects of environmental pollutants is gaining acceptance. These cells may also serve to indicate whether transient exposure to pollutants may have lasting, possibly genetic, effects, and it is toward this point that the experiments described below were undertaken.

In vitro exposure of human peripheral lymphocytes to various carcinogenic and mutagenic chemicals, as well as radiation, will elicit a DNA repair response the magnitude of which varies among individuals and with the chemistry of the particular agent (Lieberman et al., 1971; Clarkson and Evans, 1972). The exact relationship of this phenomenon, except in certain cases, to in vivo carcinogenesis by the chemicals or radiation remains obscure. However, it is firmly established that a relationship does exist (Friedberg et al., 1979).

Experimental animals are normally the subjects for test materials but in the preliminary studies described here, peripheral lymphocytes of human blood were used.

METHODS AND MATERIALS

Lymphocytes were isolated from freshly drawn, heparinized venous blood using a Ficoll/Hypaque gradient. After washing with Hank's balanced salt solution (GIBCO), the lymphocytes were resuspended in Dulbecco's minimum essential medium (GIBCO). Aliquots of 0.1 ml containing a known number of cells were dispensed into the wells of Microtiter plates (Falcon Plastics, Oxnard, California). To begin the experimental treatment, an additional 0.1 ml that contained the appropriate reagent mixture was added to the wells. DNA repair synthesis (DRS) was measured as the stimulation of incorporation of ^{3}H-TdR into cellular DNA in the presence of 10 mM HU and the DNA damaging chemical. The specific chemicals are given in the Table legends. Replicative DNA synthesis was measured as the incorporation of ^{3}H-TdR into DNA in the absence of HU. After incubation for 3 hr at 37°C the lymphocytes were collected by filtration onto glass fiber filters, washed exhaustively (15 times) with distilled water and the radioactivity retained on the filters determined by scintillation counting.

RESULTS AND DISCUSSION

Effect on DRS of Cell Number per Well

In order to establish conditions for the DRS assay, the effect of cell number per Microtiter well was measured using two agents, 4NQO (10 μM) and MMS (1 mM). Table 14 summarizes the results. With the exception of the values obtained with 0.38×10^5 cells per well, DRS increased in proportion to the number of cells per well. However, the maximum DRS per cell was seen at 0.75×10^5 cells per well. DNA replicative synthesis was measured in cells not treated with either chemical and was independent of the number of cells per well over the range tested. In this experiment, the 4NQO and MMS

were present in the Microtiter wells throughout the incubation period. Note that the extent of DRS stimulated by the agents differs. The difference in the extent of DRS is presumed to be the result of different kinds of DNA damage produced by each.

Effect of Mitogen Stimulation on DRS in Lymphocytes

A number of laboratories have reported that incubation of lymphocytes with a mitogen such as phytohemagglutinin (PHA) increases the DRS response to a test agent (Lavin and Kidson, 1977; Darzynkiewicz, 1971). This has been found in the present experiment using two DNA damaging chemicals, 4NQO and nitrogen mustard (HN2, Mechlorethamine). Table 15 shows the results of an experiment with HN2. In this experiment, the lymphocytes were exposed to HN2 for 30 min, collected by centrifugation and viability determined by trypan blue exclusion. The cells were then distributed to the Microtiter plate wells on the basis of 10^5 viable cells per well. PHA stimulation greatly increased the DRS response to HN2 up to fifty fold (at 100 μM HN2). At 1 mM, HN2 was extremely toxic to the cells and resulted in cell death.

Table 14. DNA repair synthesis in human peripheral lymphocytes: Effect of cell number per Microtiter well*

Cell Number Per Well	DNA Repair Synthesis: 10 μM 4NQO	1 mM MMS	DNA Replicative Synthesis
0.38 x 10^5	41 ± 9	17 ± 3	75 ± 14
0.75 x 10^5	228 ± 51	131 ± 32	177 ± 42
1.5 x 10^5	328 ± 46	101 ± 12	374 ± 14
3.0 x 10^5	496 ± 15	212 ± 23	603 ± 41
Expressed as CPM per 10^6 cells:			
0.38 x 10^5	1079 ± 237	447 ± 79	1974 ± 368
0.75 x 10^5	3040 ± 680	1747 ± 427	2360 ± 560
1.5 x 10^5	2187 ± 307	673 ± 80	2493 ± 93
3.0 x 10^5	1653 ± 50	707 ± 77	2010 ± 137

* Values are ^{3}H-CPM ± S.D. based on 6 replicate samples.

Table 15. DNA repair synthesis in peripheral lymphocytes before and after induction of cell replication with PHA*

	Control	10 μM HN2	100 μM HN2	1 mM HN2
Freshly Isolated Lymphocytes				
1.5 hr Incubation				
DNA Replication	3170 ± 300	2730 ± 153	1150 ± 114	520 ± 248
DNA Repair Synth.	0	340 ± 84	500 ± 54	480 ± 152
3.0 hr Incubation				
DNA Replication	6190 ± 463	4540 ± 373	1560 ± 98	600 ± 87
DNA Repair Synth.	0	560 ± 101	810 ± 104	280 ± 75
Lymphocytes Incubated 96 hr with PHA				
1.5 hr Incubation				
DNA Replication	9.01×10^5	3.68×10^5	6.18×10^4	Not Done
DNA Repair Synth.	0	2982 ± 464	19324 ± 883	Not Done
3.0 hr Incubation				
DNA Replication	1.54×10^6	6.18×10^5	1.20×10^5	Not Done
DNA Repair Synth.	0	7121 ± 760	42273 ± 10158	Not Done

* All cells were incubated with HN2 for 30 min. Incubation is time incubated with ^{3}H-TdR after HN2 treatment. Values are based on six replicates and where indicated are ± S.D. and are expressed as CPM/10^6 viable cells.

Comparative Effectiveness of Various Agents in Inducing DRS

The values of DRS stimulated by various chemicals can be quantitatively compared to give some indication of the relationship of DNA damage to DRS. Table 16 presents representative values of DRS induced in freshly isolated lymphocytes by four chemicals, 4NQO, MMS, HN2 and mitomycin C (MMC). The differences in DRS are probably due to the chemical and physical natures of the interactions of the chemicals with DNA. For example, 4NQO binds chemically to DNA and presumably causes a distortion of the double stranded DNA which then stimulates the repair enzymes. MMS breaks down under physiologic conditions to become a methyl group donor. Some sites of methylation, e.g. O^6-guanine, may stimulate DRS while other sites, e.g. N^7-guanine, may not (Pegg, 1977).

Table 16. Comparison of four DNA damaging agents for their DRS stimulating action

Chemical	Optimum Concentration	Approximate DRS During a 3 hr Incubation (^{3}H-CPM/10^6 Cells)
4NQO	10 μM	2000 - 3000
MMS	1 mM	1000 - 2000
HN2	100 μM	800
MMC	10 μM	100

HN2 may be either an alkylating agent as an ethyl donor or a crosslinking agent between DNA strands. The latter damage may be very slowly repaired. In the case of MMC, the damage to DNA is largely due to crosslinking of strands. This chemical is very effective in producing sister chromatid exchanges which are thought to be due to unrepaired damage, but is very poorly effective in stimulating DRS.

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